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Promoters largely determine the efficiency of repressor action

(RNA polymerase/repressor competition/operator position/*lac* operon)

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ABSTRACT **Operator sequence and repressor protein regulate the activity of the *lac* promoter over a >1000-fold range. Combinations of the *lac* operator with other promoter sequences, however, differ vastly in the level of repression. The data presented show that the extent of repression is determined largely by the rates of complex formation of the competing systems operator–repressor and promoter–RNA polymerase and by the rate at which RNA polymerase clears the promoter. Moreover, up to 70-fold differences in the level of repression were found when the operator was placed in different positions within the promoter sequence. A kinetic model is proposed that explains the observed effects and that allows predictions on promoters controlled by negatively acting elements.**

The *lac* repressor, though present in only 10–20 copies per cell (1), is capable of reducing the expression of the *Escherichia coli lac* operon by a factor of ≈1000 (2). When placed on a multicopy plasmid the *lac* promoter (P_{lac}) can be controlled to the same extent, provided that the intracellular repressor concentration is adjusted properly (3, 4). Under identical conditions the *lac* promoter mutant $P_{lacL8.UV5}$ (5), which is homologous to P_{lac} throughout the entire operator region and which is about equally active *in vivo* (6), is repressed an order of magnitude less efficiently (4). Moreover, P_{tac} (7), a derivative of $P_{lacL8.UV5}$ and ≈3 times stronger *in vivo* than P_{lac}, is reduced in its activity by a factor of only 50 by *lac* repressor (4). These observations indicate that the extent of repression is neither determined by the interaction between repressor and operator alone nor correlated with promoter strength *in vivo*. Additional parameters evidently play a significant role in a functionally optimized promoter/operator system.

We have studied various promoter/operator combinations by analyzing their repression *in vivo* as well as the kinetics of their interaction with repressor and RNA polymerase (RNAP), respectively. Our data show that the kinetic parameters of the RNAP–promoter interaction as well as the position of an operator within the promoter sequence drastically affect the occupancy of the operator by its repressor, which ultimately defines the efficiency of repression.

MATERIALS AND METHODS

DNA, Plasmids, and Strains. All promoter/operator sequences were obtained by total or partial synthesis and flanked upstream by a *Xho* I restriction site and downstream by an *Eco*RI site. Upon insertion into plasmids pDS2 (6) and pML3 (4) these promoters direct the transcription of the dihydrofolate reductase (*dhfr*) or the β-galactosidase (*lacZ*) coding region, respectively. For monitoring the derepressed promoter activity, *E. coli* DZ291 was transformed with pDS2 constructs. Transcriptional activity under repressed conditions was determined in DZ291 cells containing either the compatible plasmid pDM1.1 or pDM1/cI. The pDM1 plasmids (4) contain the P15A replicon and a marker conferring kanamycin resistance. Strains harboring pDM1.1 contain about 5000 copies of *lac* repressor per cell during logarithmic growth (4). Plasmid pDM1/cI produces the phage λ repressor cI under the control of $P_{lacL8.UV5}$.

Determination of Promoter Activity. The *in vivo* activities of promoters were determined by monitoring the rate of RNA synthesis whereby the β-lactamase promoter (P_{bla}) served as internal standard as described in detail by Deuschle *et al.* (6). Promoter activities below 0.3 P_{bla} units were determined by measuring β-galactosidase activity (8). Both the rate of RNA synthesis and the activity of β-galactosidase were determined in logarithmically growing cultures at OD_{600} = 0.6. Such cultures were grown at 37°C in minimal medium containing 0.1× LB (8).

Proteins. RNAP preparations were either prepared according to Burgess and Jendrisak (9) or purchased from Pharmacia Freiburg. *lac* repressor was purified as described (10).

Dissociation of *lac* Repressor–Operator Complexes. A mixture of end-labeled fragments carrying the promoter/operator constructs was allowed to equilibrate with a stoichiometric amount of *lac* repressor (5 nM) for 10 min at 37°C in 1 mM $MgCl_2$/50 mM NaCl/0.1 mM EDTA/0.1 mM dithiothreitol/2.5% (vol/vol) dimethyl sulfoxide/10 mM Tris·HCl, pH 7.6 (assay volume, 100 μl). A 100-fold excess of unlabeled operator DNA was added before aliquots were withdrawn at different times and subjected to nitrocellulose filtration. The adsorbed complexes were eluted and analyzed by PAGE and autoradiography (11).

Association of *lac* Repressor with Promoter/Operator Sequences. Mixtures of end-labeled fragments (0.2 nM each) carrying the promoter/operator constructs were exposed to increasing but limiting amounts of *lac* repressor under the above conditions. After 30 sec the samples were analyzed as described above.

Association of RNAP with Promoter/Operator Sequences. Mixtures of DNA fragments (0.5 nM each) containing the promoter/operator constructs were exposed to increasing but limiting amounts of RNAP for 2 min at 37°C in 10 mM $MgCl_2$/120 mM KCl/0.1 mM EDTA/0.1 mM dithiothreitol/5% (vol/vol) glycerol/10 mM Tris·HCl, pH 7.6 (ratio of RNAP to promoter as indicated; assay volume, 60 μl). After addition of a 10-fold excess of single-stranded competitor DNA, specific complexes were monitored as described (11).

Calculation of Rate Constants. Autoradiograms were quantified by densitometry and the rate constants were determined from the fraction of bound repressor–operator or RNAP–promoter complexes, as described (11).

RESULTS

Experimental Strategy. *lac* operator sequences were combined with promoters differing widely in *in vivo* strength and

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Abbreviations: RNAP, RNA polymerase; CRP, cAMP receptor protein.

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in the way they interact with RNAP *in vitro*. The operator sequences were placed in three different regions of the promoters, yielding the following basic constructs (Fig. 1). *O3* sequences contain the 29-base-pair (bp) wild-type operator in a position homologous to P_{lac}; the transcripts produced by these promoters are identical. *O4* sequences carry a 17-bp core region of the wild-type *lac* operator as spacer between the −10 and −33 hexamers of the promoter. *O5* sequences were obtained by fusing promoters with a 21-bp *lac* operator sequence at position −37. In addition, two *O4* constructs were prepared with λ operator O_{L1}.

The *in vivo* activities of all promoter/operator sequences were determined under repressed and derepressed conditions by quantifying either the rates of RNA synthesis or, whenever promoter activities fell below 0.3 unit of the internal standard (P_{bla}), by monitoring β-galactosidase. The activity of this enzyme correlated linearly with transcriptional activity over a 20-fold range, whereby 1 P_{bla} unit corresponds to 5000 β-galactosidase units. Furthermore, we have determined the rates of complex formation and dissociation for the repressor–operator and RNAP–promoter interactions of all constructs *in vitro* by using end-labeled isolated DNA fragments and purified *lac* repressor or RNAP.

Promoter-Specific Parameters Affecting Repression. When the transcriptional activities of promoter/operator sequences of the *O3* type were examined *in vivo* under repressed and derepressed conditions, up to 200-fold differences in the repression factor were observed among the various constructs (Table 1). Interestingly, there exists no obvious correlation between promoter strength in the derepressed state and repressibility of the different sequence combinations. For example, as mentioned above, P_{lac} and $P_{lacL8.UV5}$, two promoters of almost equal strength *in vivo* (5.5 and 3.4 P_{bla} units, respectively), differ in their repression by a factor of 10 (Table 1). Moreover, $P_{A1/O3}$, with 22 P_{bla} units the most active promoter within this group, is repressed by a factor of 27, whereas the activity of $P_{N25/O3}$ (7.7 P_{bla} units) is reduced by a factor of only 5 under identical conditions. Similar observations hold for other promoter/operator constructs of this group (Table 1).

All these sequences bind *lac* repressor with the same rate and form complexes of identical stability (Fig. 2 *A* and *B*; Table 1). By contrast, the rates of complex formation (k_{ON}) between RNAP and the various promoter/operator constructs differ more than 100-fold (Fig. 2*C*; Table 1), indicating an inverse correlation between k_{ON} of the RNAP–promoter interaction and the repressibility of the system.

Effects of Promoter/Operator Topography. In *O4*- and *O5*-type constructs, *lac* operator sequences were inserted into promoter regions known to harbor operators in several other operons (14, 15). In both sequence families, *lac* repressor reduces the transcriptional activity *in vivo* to various extents. However, whereas the repressibility of promoters of the *O5* type resembles that observed with *O3* constructs, the centrally located 17-bp operator (*O4* constructs) causes a 50- to 70-fold greater repression (Table 1).

Again there is no correlation between repressibility and promoter strength *in vivo*. Instead, from the various kinetic parameters of the *in vitro* interaction (Fig. 2 *D–F*; Table 1), it is evident that the repression factor of *O4* and *O5* constructs correlates inversely with the rate of complex formation between RNAP and promoter (Table 1). Moreover, the repression of promoters containing a single λ operator follows the same pattern: a derivative of coliphage λ promoter P_L carrying just O_{L1} is repressed by a factor of 100 by repressor cI, whereas $P_{A1/OL1}$, which contains the same operator in the homologous position (Fig. 1) and which binds RNAP 4 times more rapidly, is reduced in its activity by a factor of only 25 (Table 1).

Thus, the position of the *lac* operator within a promoter sequence profoundly affects the repression of promoter activity. Within each group of promoter/operator constructs, however, the rates of complex formation of the two competing systems seem to largely determine the level of repression.

Influence of Complex Stability on Repression. All promoter sequences used in this study bind RNAP stably enough ($t_{1/2} \geq$ 10 min; refs. 4 and 11) to ensure an irreversible process in the presence of nucleoside triphosphates. Thus, an effect of RNAP–promoter complex stability on repression is not expected and in fact was not found (data not shown). By contrast, it is well established that the half-life of *lac* repressor–operator complexes affects repression levels (16).

Although the sequence families *O3–O5* bind repressor with the same rate (5×10^9 $M^{-1} \cdot sec^{-1}$; Fig. 2 *B* and *E*), the resulting repressor–operator complexes differ slightly in their stability (Fig. 2 *A* and *D*; Table 1). All sequences containing the 29-bp operator (*O3* constructs) bind *lac* repressor to form complexes with a half-life of 4 min at 50 mM NaCl. Under the

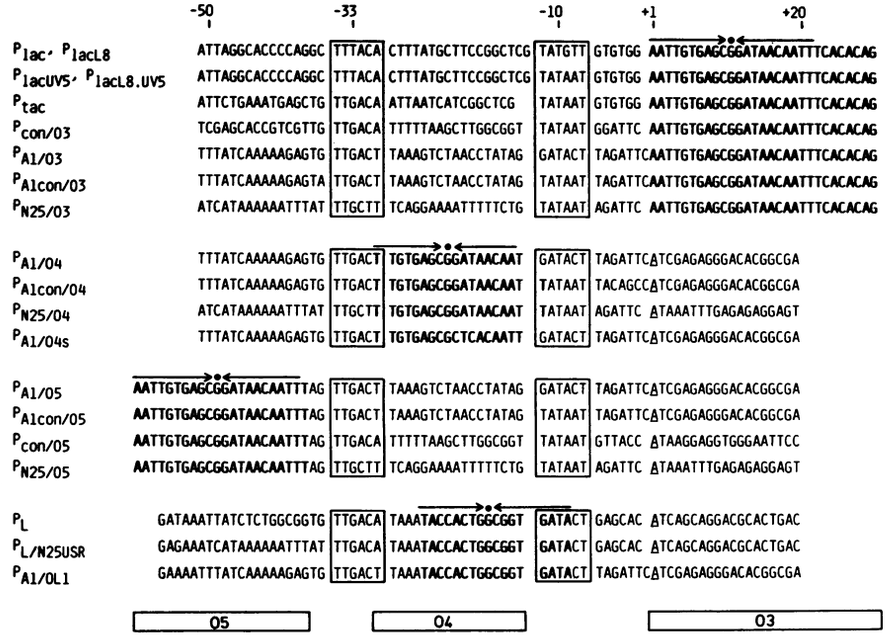

FIG. 1. Promoter/operator sequences. The sequences are aligned at the conserved hexamers and at the first nucleotide transcribed (+1). The central base pair (●) and the dyad symmetries (arrows) within the operator sequences (heavy letters) are indicated. The origin and the *in vivo* activity of the natural promoters were described (6). P_{lac}UV5 and P_{lac} are, unlike P_{lac}L8 and P_{lac}L8.UV5, stimulated by the cAMP–CRP complex (where CRP is the cAMP receptor protein) (5, 12). P_{A1con} is derived from P_{A1} of coliphage T7 and contains a consensus −10 hexamer. $P_{A1/O4s}$ contains an operator with a symmetrical sequence. Two *O4*-type operator/promoter sequences containing the λ operator O_{L1} were obtained by replacing the upstream region (USR) of P_L with the corresponding sequence of P_{N25} and by properly converting the spacer region of P_{A1}. The resulting constructs were designated $P_{L/N25USR}$ and $P_{A1/OL1}$.

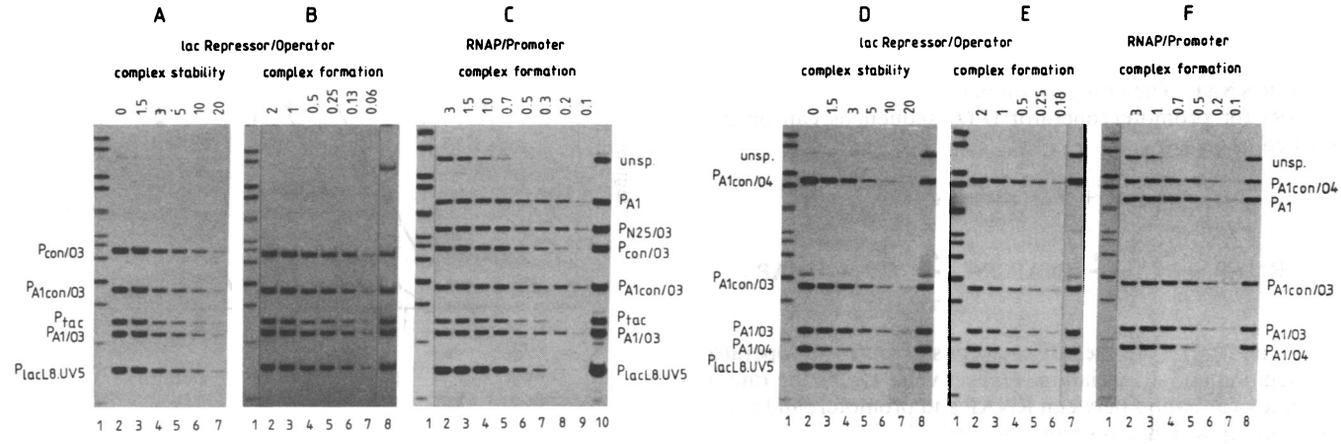

FIG. 2. Kinetic analyses of complexes formed between promoter/operator sequences and *lac* repressor or RNAP. (*A* and *D*) Dissociation of preformed *lac* repressor–operator complexes was monitored in the presence of an excess of unlabeled operator DNA. At the times indicated (in min above each lane), aliquots were withdrawn and subjected to nitrocellulose filtration. The adsorbed complexes were eluted and analyzed by PAGE and autoradiography (called NC assay). The promoter/operator sequences within the fragment mixture are indicated. A non-operator fragment is denoted unsp. (*B* and *E*) Association of *lac* repressor with promoter/operator sequences. Mixtures of end-labeled fragments containing the promoter/operator sequences indicated were exposed to increasing amounts of *lac* repressor. After 30 sec, the samples were analyzed by the NC assay. The ratios of repressor to operator are indicated above each lane. (*C* and *F*) Association of RNAP with promoter/operator sequences. Mixtures of DNA fragments containing the indicated promoter/operator sequences were exposed to increasing amounts of RNAP. After addition of an excess of competitor DNA, specific complexes were monitored by the NC assay. The ratios of RNAP to promoter are indicated above each lane.

same conditions the half-life of complexes between repressor and *O5* sequences is only 2 min. When *lac* repressor binds to the 17-bp core region, as in the *O4* constructs, the half-life of the resulting complexes depends on neighboring sequences. Among our constructs it varies between 1.4 min for $P_{A1/O4}$ and 3.5 min for $P_{A1/O4s}$ (Table 1). Surprisingly, this significant difference has only a marginal effect on the repression factor of the latter two sequences (≈25%; Table 1). As will be discussed later this is most likely due to the lower rate by which RNAP clears $P_{A1/O4s}$. Thus, the contribution of the half-life of the repressor–operator complex to repression can only be assessed when all the parameters of a promoter/operator system are comparable.

Does RNAP Displace Repressor from a Preformed Repressor–Operator Complex? DNase protection ("footprinting") analysis has shown that RNAP binds to promoter/operator sequences of the *O3* and *O5* type in the presence of prebound repressor (4). To examine whether this affects the stability of the repressor–operator complex, the half-life of $P_{N25/O3}$–repressor complexes was determined in the absence or presence of RNAP by using end-labeled, operator-carrying DNA fragments as competitors. As shown in Fig. 3*C*, the

Table 1. Promoter activities and kinetic parameters of various promoter/operator sequences

Promoter	Promoter activity: Repressed β-Gal units	Promoter activity: Repressed P_{bla} units	Promoter activity: Derepressed P_{bla} units	Repression factor	RNAP/P $k_{ON} \times 10^{-8}$, $M^{-1}\cdot sec^{-1}$	R/O $t_{1/2}$, min
P_{lac}	30 ± 5	0.006 ± 0.001*	5.5 ± 1.0	920 ± 230	0.02 ± 0.01†	4.0
P_{lacL8}	15 ± 8	0.003 ± 0.001*	0.4 ± 0.1	150 ± 30	≈0.002†	4.0
$P_{lacL8.UV5}$	150 ± 10	0.03 ± 0.01*	3.4 ± 0.5	110 ± 40	0.11 ± 0.02	4.0
P_{lacUV5}	2,740 ± 350	0.55 ± 0.02	9.3 ± 1.3	17 ± 3	ND	4.0
P_{tac}	1,510 ± 170	0.36 ± 0.02	17.6 ± 1.8	50 ± 6	0.85 ± 0.09	4.0
$P_{con/O3}$	3,740 ± 180	0.70 ± 0.10	11.8 ± 2.0	17 ± 4	1.1 ± 0.2	4.0
$P_{A1/O3}$	4,180 ± 530	0.83 ± 0.10	22.2 ± 3.0	27 ± 5	1.5 ± 0.2	4.0
$P_{A1con/O3}$	8,890 ± 490	1.80 ± 0.10	11.9 ± 2.0	7 ± 2	2.6 ± 0.3	4.0
$P_{N25/O3}$	8,160 ± 450	1.50 ± 0.10	7.7 ± 1.3	5 ± 2	2.9 ± 0.3	4.0
$P_{A1/O4s}$	58 ± 3	0.011 ± 0.001*	25.1 ± 1.3	2,200 ± 240	0.6 ± 0.05	3.5
$P_{A1/O4}$	110 ± 2	0.022 ± 0.001*	38.1 ± 3.4	1,730 ± 170	0.6 ± 0.05	1.4
$P_{A1con/O4}$	220 ± 12	0.044 ± 0.003*	16.8 ± 2.0	380 ± 50	1.7 ± 0.4	2.0
$P_{N25/O4}$	250 ± 40	0.050 ± 0.002*	17.6 ± 1.0	350 ± 60	2.9 ± 0.2	2.0
$P_{A1/O5}$	13,700 ± 1300	2.7 ± 0.5	32.3 ± 3.7	12 ± 4	1.5 ± 0.2	2.0
$P_{A1con/O5}$	8,500 ± 900	1.7 ± 0.6	7.7 ± 1.2	5 ± 1	2.6 ± 0.3	2.0
$P_{con/O5}$	ND	2.5 ± 0.5	14.9 ± 2.0	6 ± 2	1.1 ± 0.1	2.0
$P_{N25/O5}$	ND	3.5 ± 0.5	14.9 ± 1.0	4 ± 1	2.9 ± 0.3	2.0
P_L	7–42	0.0014–0.008*	53.0 ± 8.0	10,000–30,000	0.14 ± 0.02	ND
$P_{L/N25USR}$	2,950 ± 370	0.60 ± 0.03	58.0 ± 9.0	100 ± 15	0.14 ± 0.02	ND
$P_{A1/OL1}$	6,400 ± 670	1.25 ± 0.07	31.0 ± 4.0	25 ± 4	0.60 ± 0.05	ND

The promoter activities under repressed and derepressed conditions are given in P_{bla} units. Where indicated (*), only β-galactosidase (β-Gal) activity was assayed and the corresponding P_{bla} units were calculated. The repression factor was calculated from P_{bla} units of the derepressed state divided by P_{bla} units of the repressed state. RNAP/P k_{ON} describes the rate of complex formation between RNAP and promoter, and R/O $t_{1/2}$ is the half-life of the repressor–operator complex. Unlike a previously published (6) value, the *in vivo* activity of P_L is 53 P_{bla} units (13).
†We assume that the k_{ON} of P_{lacL8} is equivalent to the k_{ON} of P_{lac} in the absence of cAMP–CRP; k_{ON} of P_{lac} is taken from ref. 12.

stability of the repressor–operator complex is not changed by RNAP binding.

Kinetic Description of the Competition Between *lac* Repressor and RNAP. The competition between RNAP and repressor (R) for promoter/operator (*PO*) sequences can be described as follows.

$$R + PO \underset{(k_R)_{-1}}{\overset{(k_R)_{+1}}{\rightleftharpoons}} R\text{–}PO$$

$$RNAP + PO \xrightarrow{k_{ON}} RNAP\text{–}PO \xrightarrow[NTP]{k_{CL}} PO + RNAP_{el}$$

$(k_R)_{+1}$ and $(k_R)_{-1}$ are the rate constants of repressor–operator association and dissociation, respectively; k_{ON} is the rate of complex formation between RNAP and promoter; and k_{CL} is the rate at which a promoter is cleared, resulting in a free promoter/operator sequence and a transcriptional elongation complex ($RNAP_{el}$).

At steady state the repression factor (F_R) is defined by the equilibrium constants of the repressor–operator and the RNAP–promoter interaction, respectively, as well as by the concentrations of RNAP and repressor:

$$F_R = \frac{(K_{eq})_{R/O}}{(K_{eq})_{RNAP/P}} \times \frac{[R]}{[RNAP]}.$$

At constant concentration of repressor and RNAP,

$$F_R \approx \frac{k_{CL}(k_R)_{+1}}{k_{ON}(k_R)_{-1}}.$$

Three of these rate constants—namely, k_{ON}, $(k_R)_{-1}$, and $(k_R)_{+1}$—can be measured *in vitro*, and we propose that the values determined also describe the *in vivo* situation, though not necessarily in absolute terms. A relative value for the rate of promoter clearance, k_{CL}, can be estimated from the *in vivo* promoter strength in the derepressed state. For these estimates we assume that a promoter strength of 100 P_{bla} units corresponds to one productive initiation event per second (6).

DISCUSSION

Our results suggest that within each of the three groups of promoter/operator constructs studied, the rate of complex formation (k_{ON}) between RNAP and promoter determines the level of repression. Indeed, an excellent inverse correlation between k_{ON} and repression is obtained for most of the *O3* constructs (Fig. 3*A*). At steady state the repression should, however, be determined by the equilibrium constants $(K_{eq})_{R/O}$ and $(K_{eq})_{RNAP/P}$, which reflect the occupancy of the promoter/operator sequence by the respective proteins. The occupancy of a promoter by RNAP *in vivo*, on the other hand, depends on the rate of stable complex formation as well as on the rate at which the bound enzyme clears the promoter sequence as a productively transcribing complex. Repressibility of a promoter should therefore also be affected by the latter parameter. The rate of promoter clearance, however, can differ widely among promoters and determines *in vivo* promoter strength whenever it is the rate-limiting step (11, 13, 17, 18).

Interestingly, $P_{A1/O3}$ and P_{tac}, the strongest promoters *in vivo* within this group, are repressed better than expected from their k_{ON} values, whereas P_{lacL8}, the least active promoter, is repressed rather inefficiently despite its low k_{ON} value (Fig. 3*A*). This indicates that the high rate of promoter clearance of $P_{A1/O3}$ and P_{tac}, which lowers the occupancy of the promoter by RNAP, increases the repression factor, whereas the low clearance of P_{L8} results in a less efficient repression.

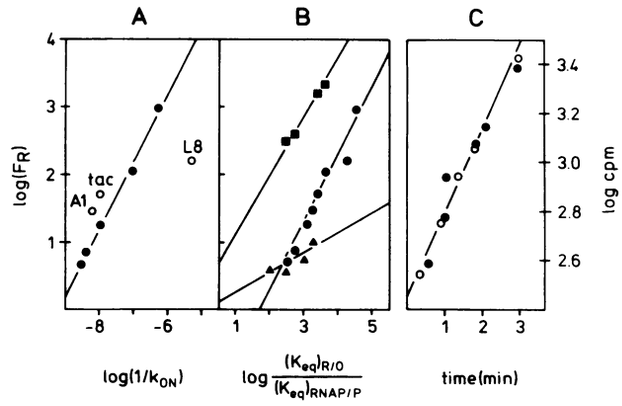

FIG. 3. Dependence of the repression factor (F_R) on various kinetic constants and dissociation of *lac* repressor–operator complexes in presence of RNAP. (*A*) The inverse of the rate of complex formation ($1/k_{ON}$) between RNAP and *O3*-type promoter/operator sequences is plotted logarithmically against the repression factor. $P_{A1/O3}$, P_{tac}, and P_{lacL8} are represented by open circles. (*B*) The repression factor for all three promoter/operator families is plotted logarithmically against $(K_{eq})_{R/O}/(K_{eq})_{RNAP/P}$ for *O3* (●), *O4* (■), and *O5* (▲) sequences. The same dependence of the repression factor on the occupancy of the operator by repressor is found for *O3* and *O4* constructs; the difference in the intercepts indicates, however, that *O4* constructs are efficiently repressed at lower repressor concentrations. The different slope found for *O5* constructs suggests a basically different mechanism. (*C*) Supercoiled plasmid DNA (0.6 nM) containing the $P_{N25/O3}$ promoter sequence was allowed to equilibrate with *lac* repressor (0.4 nM) for 10 min at 37°C in buffer containing all four nucleoside triphosphates (0.2 mM each) before RNAP (1.2 nM) was added. After 10 sec, end-labeled operator DNA was added and aliquots were withdrawn at different times and subjected to nitrocellulose filtration. The amount of retained radioactivity determined by scintigraphy was corrected for the background value. ○, Presence of RNAP; ●, absence of RNAP.

The same dependence of repression on $(K_{eq})_{RNAP/P}$ is observed for the *O4* promoter/operator sequences when the different stabilities of repressor–operator complexes in these constructs (Table 1) are taken into account (Fig. 3*B*). The repression factors determined for *O5* sequences appear less dependent on $(K_{eq})_{RNAP/P}$ (Fig. 3*B*).

The differences in intercepts and slopes in Fig. 3*B* suggest different mechanisms of repressor action depending on the position of the operator within a promoter sequence. Obviously, the *O4* arrangement allows a promoter to be controlled most tightly, whereas the *O3* and *O5* arrangements are less effective. A model that explains the observed effects is proposed in Fig. 4. Operators located in the *O3* position can be recognized by the repressor only after RNAP has cleared the entire promoter region. The two proteins will therefore simultaneously compete for their respective binding sites, and their success depends essentially upon the ratio of the two forward rate constants. By contrast, operators placed in the *O4* position are cleared by RNAP prior to the promoter sequence, giving the repressor a competition advantage over RNAP. The 50- to 70-fold greater repression factor indicates that >98% of the operator sequences are occupied by the repressor before free competition between RNAP and repressor can occur. At *in vivo* concentrations of *lac* repressor (2 μM; ref. 4) and *in vivo* rates of repressor binding as derived by Winter *et al.* (19), *lac* repressor could associate with *O4* constructs under noncompetitive conditions for 0.1–0.2 sec.

Based on these considerations, operators in the *O5* position should control transcription even more tightly. In fact, RNAP should almost exclusively encounter repressor-complexed *O5* sequences. Footprint analysis has shown that, in contrast to *O4* sequences, RNAP binds to *O5* and *O3* constructs in the presence

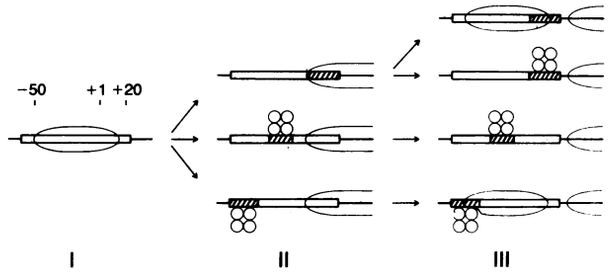

FIG. 4. Effect of operator position on repression. RNAP bound to a promoter covers ≈70 bp, from positions −50 to +20 (I). Upon productive initiation (II) of transcription, operators in *O5* and *O4* constructs are available for repressor binding before a second RNAP molecule can occupy the promoter, since the transcribing enzyme is still covering large portions of the sequence. After promoter clearance (III), RNAP and repressor compete for their respective binding sites in *O3* constructs and distribute according to their k_{ON} and the actual concentration of the free proteins. *In vivo*, >98% of the *O4* and *O5* constructs have associated with the repressor at this stage. In contrast to *O4* constructs, *O3* and *O5* sequences bind RNAP in the presence of a prebound *lac* repressor (data not shown). However, whereas productive initiation is efficiently prevented in *O3* constructs, a repressor bound in the *O5* position appears to allow initiation of transcription, though with reduced rates.

of bound repressor (4, 20, 21). However, a repressor in the *O3* position efficiently prevents the transition of the RNAP–promoter complex into a productive state, as proposed by Straney and Crothers (21) for $P_{lacL8.UV5}$, and consequently repression depends on the occupancy of the two competing sites. By contrast, with a repressor at *O5*, RNAP appears capable of rather independently initiating transcription, though at low efficiency. This is supported by the low dependence of the repression factor on promoter-specific kinetic parameters, as indicated by the slope in Fig. 3*B*.

The above conclusions were drawn under the assumption that the binding of RNAP to a promoter/operator sequence does not affect the stability of a preformed repressor–operator complex. Based on footprint analysis (4), we rule out such interference for *O4* constructs since RNAP does not bind to preformed *O4*–repressor complexes. For *O3* sequences, however, we have examined the half-lives of repressor–operator complexes in the presence or absence of RNAP and indeed no difference was observed (Fig. 3*C*).

The model proposed permits examination of the regulation of natural operons in a more quantitative manner. For example, whereas the *lac* operon is down-regulated a factor of 1000 by its repressor, the L8 mutation—which renders P_{lac} independent of CRP (5)—is repressed by a factor of only 300 (22) despite its low k_{ON} (Table 1). We propose that cAMP–CRP activates transcription at P_{lac} by increasing not only its rate of complex formation with RNAP (12) but also the rate of productive initiation, as has been shown for the *malT* promoter (23). Thus, the unexpected low repression factor observed for P_{lacL8} is due to the effect of the L8 mutation on promoter clearance. The efficient repression of the *lac* wild-type promoter in the absence of glucose, on the other hand, appears again due to the dual action of cAMP–CRP: the increased rate of promoter clearance balances the higher rate of RNAP binding by the promoter–cAMP–CRP complex. Finally, a low repression factor is predicted for $P_{lacL8.UV5}$, since in comparison to P_{lac} it exhibits a high k_{ON} while the L8 mutation prevents a positive effect of cAMP–CRP on promoter clearance.

The three promoter-specific properties pertinent for high level of repression—i.e., a low rate of RNAP binding, efficient promoter clearance, and a centrally located operator—increase the occupancy of the operator by the repressor. This allows a tight control of transcription even with short-lived repressor–operator complexes as long as the rate of repressor binding is high. Extremely large forward rate constants ($>10^9\ M^{-1}\cdot sec^{-1}$) were indeed reported for both the *lac* and the λ repressor–operator interaction (19, 24). In addition, cooperativity between multiple repressor binding sites and multimeric repressor proteins add further to the occupancy of operators while maintaining the required flexibility of the system (i.e., rapid discrimination between specific and nonspecific sites; low intracellular repressor concentration).

All these properties appear to be unified in the P_L/O_L promoter/operator sequence of coliphage λ, where the high *in vivo* activity of P_L can be repressed by a factor of $>10^4$ (Table 1). Repression factors in the same range were obtained when suitable promoter and *lac* operator sequences were combined based on the principles described above (unpublished work).

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